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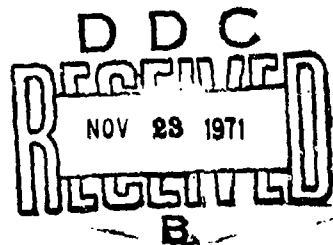
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THERMAL INACTIVATION OF VIRUSES

REPORT 1. THE RELATIONSHIP BETWEEN THE RATE OF INACTIVATION AND TEMPERATURE

A S Novokhatskiy
D I Ivanovskiy Virological Institute (Institut virusologii)
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The speed and dynamics of the inactivation of virus at various temperatures depends on multiple factors. Basic among them are the nature and structure of the viruses, their genetic peculiarities, the state of the medium in which the viral molecules are suspended, and the period and conditions of the preservation of viral preparations. However, enough stable characteristics inherent to each type of virus exist to determine the thermodynamic process for inactivating the virus. As the results of the study of various viruses showed, the mechanism of their thermal inactivation at high and low temperatures substantially differs (5,6,16,33), requiring, obviously, completely different methods of stabilizing viral preparations in relation to the level of temperature acting on them.

We tried to determine some principles of the inactivation of RNA-containing viruses under various conditions using Venezuelan Equine Encephalomyelitis (VEE), Sindbis (SV), and Vesicular Stomatitis (VSV) viruses. By basic functional tests the dynamics of change in infective activity of viruses were determined. Report No 1 gives an account of the results of investigating the constants of the rate of inactivation at various temperatures, as well as data on some thermodynamic characteristics describing the process of loss of infective activity by viruses.

Materials and Methods

VEE, SV, and VSV (Indian strain) viruses were received from the Museum of Viral Strains of the D I Ivanovskiy Institute of Virology, AMN SSSR and placed in cultures of fibroblasts of chicken embryos (FEK). All three viruses were grown in medium No 199 with a 2% heated bull serum component.

Initially trypsinized FEK was prepared by the usual method.[1]

Viral infectivity was determined by plaque titration under agar overlay according to Porterfild or Dalbekko[11,24] Cultivation of the cells and incubation of plaque forming system was conducted in an atmosphere containing 3% CO_2. Hemagglutinating activity of VEE and SV viruses was determined according to the earlier described method of Klark and Kazals.[?]

As a rule, the viruses were heated suspended in a storage medium. The viruses were titrated immediately after being heated through.

The influence of the temperature gradient on VEE and SV viruses. In dia 1 the results of heating SV and VEE viruses for 20 minutes at temperatures from 20° to 70° are set down. Each point was determined by the average of two parallel experiments. The interval of temperature from 50° to 60° was shown to be critical for the viruses studied. In the interval from 20° to 50° an increased variety of viral titers was

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noted, with each 10° rise resulting in a 0.1 lg for SV and a 0.3 lg for VEE; after 50° each 2° increase in temperature resulted in furthur inactivation in the medium of 1.5 to 2 lg. Heating for 20 minutes at 70° completely inactivated the infectivity of both viruses.

The break at the 50° point on the curve indicating change in VEE and SV infectivity in relation to temperature affirms, in all probability, the existence of various mechanisms for inactivating infectivity of the studied viruses at temperatures above and below 50°.

Dia 1. Changes in infectivity of VEE (1) and Sindbis (2) viruses during heating for 20 minutes at various temperatures. Along the y-axis - activity of the virus in lg EOE/ml; along the x-axis - temperature in degrees.

For more detailed study of these processes a special series of experiments was set up.

Determination of enthalpy and entropy of heat inactivation of viruses. Viruses were incubated at temperatures from 40 to 56° and identified after a suitable interval of time with the goal of obtaining more detailed data for authentic determination of the fine dynamics of inactivation. The speed constant of inactivation was calculated according to the formula

$$k_{in} = \frac{2,303 \log C/C_0}{t}$$

with k_{in} the speed constant of viral inactivation, C - activity of virus on expiration of time and incubation, C_0 - activity of virus before incubation.

Dia 2 represents the Arrhenius equation of the logarithm of the speed constant of inactivation (lg k_{in}) of VEE virus from the inverse absolute temperature (\phi). Two components were computed, intersecting at 40° and 42° and having various angles of inclination that represent various mechanisms influencing the loss of infectivity of VEE virus during high and low temperatures. Analogous results were obtained for Sindbis virus (see dia 2 b). In dia 2 b changes in the value of the logarithm of the constant of speed of inactivation of VSV virus in the temperature range from 45° to 56° are shown.

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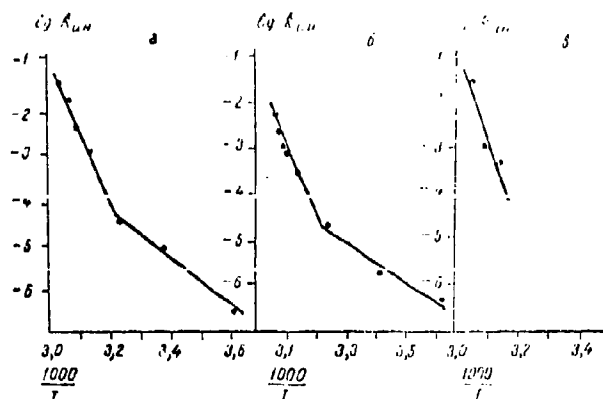
Dia 2. Graphic relationship between the logarithmic constant of inactivation from inverse absolute temperature for (a) VEE, (b) Sindbis, and (c) Vesicular Stomatitis.

According to Eyring's formula[17]:

$$\ln k_{in} = \ln\frac{kT}{h} + \frac{\Delta S}{R} - \frac{\Delta H}{RT}$$

with k_{in} the constant of speed of viral inactivation; k - constant of Boltsman (1.380 x 10^{-16}erg/degree); h - Plank constant (6,625 x 10^{-27} erg/second); T - absolute temperature; R - gas constant (1,987 calories/degree x mole); ΔH - enthalpy of activation; ΔS - entropy of activation determined for VEE virus; enthalpy and entropy of activation of the process of thermoinactivation, resulting from the two components of Arrhenius' equation. For VEE virus the sloping component is characterized by the value of ΔH_{nk} = 26,000 cal/mole and ΔS_{nk} = 3.98 UE (units of entropy), and the steep high temperature component ΔH_{pr} = 75,800 cal/mole and ΔS_{pr} = 162.8 UE. For Sindbis virus ΔH high and low temperature areas of the graph are dependent on the logarithmic constant of the speed of inactivation from the inverse absolute temperature, consisting of 78,000 and 20,000 cal/mole. Enthalpy of inactivation (ΔH) of the vesicular stomatitis virus within the 45° to 56° range was determined to be about 81,000 cal/mole.

Dia 3 represents the dynamics of thermal inactivation of VEE virus at 52°. The initial period of slow inactivation of viral infectivity accompanied by gradual lowering of the hemagglutinating activity is clearly expressed. This period lasts 30 to 35 minutes and alternates with a phase of sharply accelerated inactivation. Hemagglutins in this period were not identified.

The described viral inactivation rates fluctuate in dependence on temperature, to whose influence the virus is subjected, the period of its storage, the state of the medium, the viral molecules present, and other conditions. From 37° to 38° accelerated inactivation usually is not apparent; it can be detected only after 24 to 36 hours of incubation. With raised temperature the (initial) heating stage of accelerated inactivation come earlier and earlier, while at 56°-58° it does not become fundamental to the dynamics of thermal inactivation of VEE virus.

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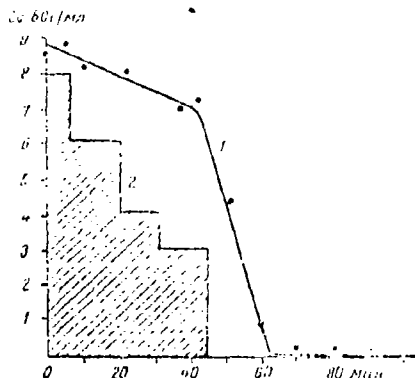
Dia 3. Dynamics of inactivation of VEE virus at 52°.
1 - lg BOE/ml   2 - lg GAE/ml

Separate determination of the constant of speed of inactivation for the described parts of the VEE virus inactivation curve gave two rows of different indications. The significance of lg k_{in} in this case lies in the course of earlier determinations (see dia 2 a) of components of Arrhenius' equation, completing the picture of crosssover (dia 4 a). The value of lg k_{in} for the periods of slow inactivation of virus remained a sloping, low temperature component of the graph, but for the periods of accelerated inactivation there was an abrupt, high temperature component of the graph with lg k_{in} dependent on T.
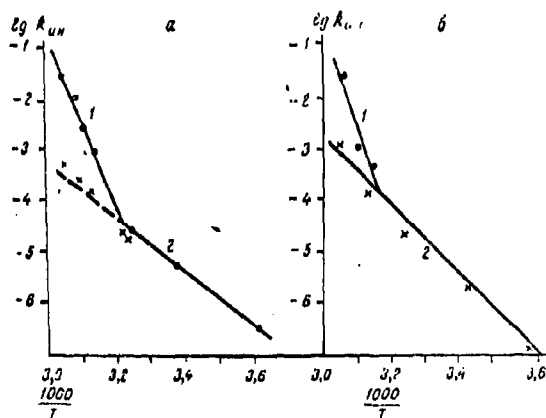
Dia 4. Graph dependence of logarithm of constant of inactivation on inverse absolute temperature.

Arrhenius' equation: a, determined separately for (1) protein and (2) nucleic types of thermal inactivation of VEE virus; b, for the process of thermal inactivation of Vesicular Stomatitis virus determined (1) experimentally and (2) according to data on the time of polyinactivation according to Galasen.[13]

The stabilizing influence of the Mg^{2+} cation. To viruses suspended in a storage medium we added equal volumes of 25% solution of magnesium sulfate or physiological solution. Then we maintained viral suspension at various temperatures titrating the virus immediately after the end of heating.

Dia 5 a,b,c represents the influence of magnesium sulfate on the thermal stability

of viruses at 50°. The greatest stabilizing effect was on VSV, the least on VEE. There has been interest in the fact that stabilizing action of magnesium sulfate appears at temperatures above 37° and concerns periods of accelerating viral inactivity which are described by characteristic high temperature components of Arrhenius' equation. More detailed peculiarities of influence of various factors on the durability of viruses under such conditions will be seen in another report.

Discussion

Existence of two types of inactivation - low temperature characterized by lower enthalpy and entropy of activation values and high temperature for which the values were several times greater - has been noted by various authors for a variety of viruses according to their nature. Data on these investigations presented in Table 1 show that the presence of two principle types of heat inactivation are general for viruses, reflecting their nucleoprotein nature.

TABLE 1

THERMODYNAMIC CHARACTERISTICS OF HEAT INACTIVATION OF SOME VIRUSES

Virus	ΔH_{pr} (in cal/mole)	ΔS_{pr} (in UE)	ΔH_{nk} (in cal/mole)	ΔS_{nk} (in UE)	temp (in degrees) crossover	bibliographic reference
Phage						
T1	95	207				23
T2	72	139				23
T3	105	246				23
T4	131					4
T5	73.2	169				20
T7	77					4
M1	76	165				12
M2	87	195				12
M4	136	347				12
Streptophage	76	165				9
Stafilophage	137		14			8
VTM	192	459	19	-20		25
RNA-VTM			19-13.5	-11-26		14
Polio virus	244	689	28	7	44	33
Coxsackie	200					26
Foot and Mouth	116-175	291-460	30-45	23-33	42-53	5
	120.6		27.2		43	6
Rhinovirus	100	242	19	-18	39	33
Adenovirus	50					15
Vaccines			14		37	27
Cytomegali	55.7-92.4		12.5-13.5		30-37	32
Respiratory-syncytial			4	-68		16
Semliki			11-11.5	37.5		19
Cori	70		18	-30		7
Rous sarcoma	77.9		19.9		45	10
VEE	75.8	162.8	26	3.98	40-42	Author's data
Sindbis	78		20			
Influenza						
A-hemagglutins	170	450				30
B-hemagglutins	340	950				
NDV-hemagglutins	125	300				
NDV virus	125	300	29	30		31

5.

TABLE 2

Dependence of enthalpy and entropy of high temperature inactivation
of DNA-containing viruses on molecular weight of DNA

Virus	Mol wt of virus x 10^6 Dalton	Mol wt of viral DNA x 10^6 Dalton	ΔH_{pr} (in cal/mole)	ΔS_{pr} (in UE)
T3		35-49	105	246
T1	140	45	95	207
T5	145	80	73.2	169
T2	250	120-130	72	139

TABLE 3

Energy of activation of thermal inactivation of lyophil viruses

Virus	ΔH (in cal/mole)	ΔS (in UE)	Bibliographic Reference
FAG T1	27.5		22
FAG T3	19.1		
FAG T7	12.7		
Polio	28.8	21	18
	20.8	-10	

Analyzed systematically, the material in Table 1 can be divided into two
viral groups. In the first group are DNA-containing viruses for which a fairly
clear inverse proportional relationship vetween ΔH_{pr} and ΔS_{pr} on one siee and the
molecular weight of the virus and the viral DNA on the other (Table 2) develops.
The second group consists of viruses characterized by relatively high values for
ΔH and ΔS.

The remaining viruses have various values of enthalpy and entropy of activation
and systematizing them according to this indication is especially difficult. It is
interesting to note that if the relationship $\Delta H_{pr}/\Delta S_{pr}$ consists of 2-2.5, then the
relationship between H_{nk} and S_{nk} is more complex, but as is evident from Table 1,
a straight proportional dependence is rather durably preserved. During determination
of the energy of activation of lyophilic preparations of viruses, the low value of
ΔH and ΔS even with the temperature around 70° to 90° (Table 3) was discovered.

Sharp change in speed and character of VEE and Sindbis viruses inactivation
at temperatures over 50° is noteworthy in regard to temperature characteristics
for the beginning of heat denaturing of protein. Values of ΔH_{pr} and ΔS_{pr} fully
supported this hypothesis. Dimmok[33] showed with a serological reaction that during
high temperature inactivation of polio and rhinovirus the structures of their pro-
tein coatings are disturbed.

If on breaking one hydrogen bond 5000 cal/mole of energy of activation and 12
enthalpic units[28] are required, then it can be shown with sufficiently good conformity
that, using the values we extracted, ΔH_{pr} and ΔS_{pr} agree and demonstrate that the

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denaturing of VEE protein molecules is implemented by breaking 14 to 15 hydrogen bonds.

The maximum ΔH_{nk} and ΔS_{nk} values for low temperature inactivation of VEE and Sindbis viruses allows one to determine that loss of infectivity in this case comes from viral RNA destruction. This emphasizes the results of Ginov and associates[14] in the development of laws of thermal inactivation of nucleic acid.

Concurring data was obtained for several other viruses.[5,10,33] A break in the graph dependence of $lg\ k_{in}$ on $(\frac{1}{T})$ shows the level of temperature at which the influence of a given mechanism of inactivation becomes dominant. Dia 4 a shows that for VEE virus at 60° - the temperature of separation of infective nucleic acid - the speed of inactivation of RNA will be 100 times lower than the speed of denaturing of virus proteins.

The level of transfer of the high and low energy components of Arrhenius' equation is determined by various conditions, important among them being the state of the medium in which the virus is suspended, as well as properties of viral molecules. In one paper[5] data on thermal inactivation of various strains of foot and mouth disease is presented. With increased thermal stability of this strain, transfer from nucleic to protein type of inactivation occurs at ever high temperatures, with this strain attaining the greatest stability at 53°. During heating to 45° - 56° of the strain of Vesicular Stomatitis virus we studied, the energy of activation was about 80,000 cal/mole, that is, characteristic protein type inactivation; at the same time, the value of poluinactivation of the virus was computed, according to Galasso[13], with the result that the energy of activation was found to be, in the range from 4° to 56°, about 36,400 cal/mole, a more naturally nuclear type of inactivation (see Dia 4 b). The described divergence is explained, in all probability, by the variety of virus strains used.
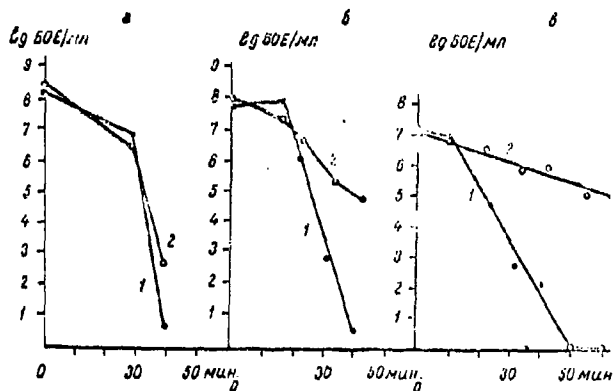
Dia 5. The stabilizing influence of Mg^{2+} on the thermal inactivation of a. VEE, b. Sindbis and c. Vesicular Stomatitis viruses.

1 - control; 2 - inactivation in the presence of a 1 M solution of $MgSO_4$

In 1965 Hollis and co-authors[29] reported that a 1 M solution of magnesium sulfate did not raise the durability of vesicular stomatitis and Sindbis viruses at 50°, that it was a mistake explained by a series of circumstances. The results of our investigation show that magnesium sulfate results in a stabilizing influence on studied viruses during heating at 50°. However, the stabilizing effect is related only to the protein type of inactivation, which for these viruses does not always appear from the first minute of heating. Data presented in Dia 5 show the presence of the beginning period, most apparent with VEE virus (see Dia 3), during which inactivation is predominately nuclear, over which a 1 M solution of magnesium sulfate has no influence. This is emphasized by the results of the study of the influence of magnesium sulfate at low temperatures. Titers of VEE and SV viruses stored a long time in the presence of magnesium at 4° did not differ from the control.

According to Klourayt and Parker,[21] American swine fever virus at 4° remained stable for months in a medium free of Ca^{2+} and Mg^{2+} ions, which, in this way, showed no substantial influence over the nuclear type of viral inactivation.

It can be surmised that Mg^{2+} ions, creating lengthened valent bonds, increase the stability of protein molecular coatings of viruses, raising, in this way, their thermal stability.

Opinion differs on the question of the influence of ΔH and ΔS on viruses. One investigator considered that the state of the medium and pecularities of strains of viruses influence the level of disposition of high and low temperature components on the graph of Arrhenius' equation and the temperature, during which transfer occurs, not essentially changing the slope of the line; that it, the energy value of activation.[5,19] Others think that the energy value of activation can change within wide limits, in particular for polio and rhino viruses under the influence of $MgCl_2$.[37]

Results of the detailed investigation of the laws of viral inactivation at various temperatures permit substantial conclusions concerning the factors determining preservation or loss of the infective state of viral particles, on the mechanisms of thermal stability of viral preparations, as well as on change in structure of the organization of virions in the thermal inactivation process. Determination of thermal dynamic laws and parameters is absolutely necessary during the study of materials and conditions influencing the thermal quality of viruses. Efficient investigations in this area hold the possibility of selective breeding of stabilizing factors on the basis of precise characteristics.

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